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A discussion of immune tolerance and the layered immune system hypothesis

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Is There a Special Requirement for Treg During the Fetal Period?

CC Anderson, critique. Essentially, the question being posed in the review of Mold and McCune[1] is whether the fetal/neonatal immune system has special properties that predispose it to tolerance. Why do antigens introduced early in life induce tolerance? The authors' answer is the "layered immune system" that mechanistically involves a preponderance of Treg early in life. This reviewer agrees that there are likely to be differences between the fetus and adult that help the fetus establish tolerance and immune system homeostasis. The authors identify some of these factors and others are likely to be found with further studies. Even our own studies have suggested a factor that may help in the establishment of tolerance in the fetus/neonate, with the lack of mature lymph nodes restraining homeostatic activation when T cells first seed the periphery.[2] Nevertheless, my main question is whether these differences between the fetus and adult are central to the question of how the decision between immunity and tolerance is made.

Jeff Mold, response. As stated by Colin above, we and others have recently shown that in humans there is a preponderance of Treg early during development, something that has not been seen in any small rodent models. Tregs are present in high frequencies at the very earliest stages of peripheral T cell colonization in humans (15–20% of all $CD4^+$ T cells in the fetus as compared with 4–7% in the neonate and adult).[3-5] In the most extreme cases, an absence of these cells results in failure of the fetus to survive in utero and in milder cases death shortly after birth.[6] I believe that this population of cells exists to establish a dominant form of tolerance to many antigens that the fetus can encounter during development and to prevent potentially disastrous inflammatory responses from developing in utero. Such a mechanism provides a sufficient explanation for the widely demonstrated phenomenon of an early developmental window where tolerance is achievable after which immunity prevails. Moreover the existence of a layered immune system (a phenomenon first proposed by Leonard and Leonore Herzenberg in 1989)[6] where the first layer is biased toward tolerance, offers a simple explanation for the different pathologies observed in fetal and neonatal infections as well as the different outcomes following vaccinations at early ages.[6,7]

CC Anderson, response. Clearly these "Treg" cells are important, the question is whether they are specifically important during a putative "window of tolerance" in early ontogeny, and whether they are the arbiters of self vs. nonself. Absence of Treg later in adult life, similar to a lack of these cells during fetal/neonatal life, leads to lethal inflammatory disease.[8] Together these data do not argue for any special window of time where Treg are needed for dominant tolerance (or more accurately, increased thresholds for activation), there is nothing special about the fetal/neonatal period is this regard, at least in a qualitative sense. The critical "rules" of self/nonself discrimination for the fetal/neonatal immune system are the same as for the adult; however, there are quantitative differences that may come into play. The absence of Treg, whether in the neonate or adult, reduces the threshold for activation of self (and foreign; i.e., they are not the arbiters of self vs. nonself) specific T cells. Since self-reactive cells are likely to be at a higher frequency in newly generated T cells (recent thymic emigrants; RTE), as RTE have yet to go through the filter of peripheral tolerance mechanisms, Treg will be important whenever RTE are present. The fetal/neonatal period has a relatively higher proportion of RTE compared with more adult stages, and one might anticipate a need for somewhat higher numbers of Treg at this stage as a consequence. However, other factors (e.g., the immaturity of lymphoid stroma) may be sufficient to compensate for higher proportions of RTE/self-reactive cells in the fetus/neonate.

Jeff Mold, response. Whether or not there are elevated autoreactive T cells present in the fetus (or neonate—which I maintain in humans is a totally different scenario), is not central to my argument, or for that matter, the differences in opinion between Colin and I regarding how immunological tolerance is generated in the fetus. I support the existence of a totally different type of T cell present in the human fetus that has different rules for how it behaves after stimulation than those observed in the adult. As we recently published, the RTEs in the fetal T cell pool are likely to behave in an entirely different way from those in the neonate or the adult making it, in my opinion, unnecessary for them to be suppressed by pre-existing Treg cells. We performed

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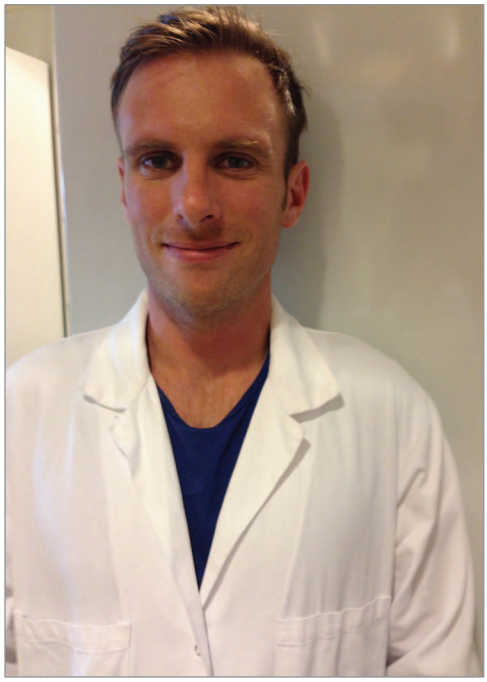

Jeff Mold

comparisons of $CD4^+CD8^-CD3^+$ thymocytes generated from fetal or adult HSPC in the same thymic environment and showed that those generated from fetal hematopoietic stem/progenitor cells (HSPC) were functionally different from those in the adult, with a propensity to become Treg upon activation.[9] This would be an example of a potential difference between the fetal and adult system with respect to the rules for self/nonself-discrimination.

I agree that the mechanisms that Colin has suggested have been established and are undoubtedly important for the maintenance of tolerance in the neonate and adult. I simply would make the addendum that in larger animals, with longer gestational periods, our immune systems exhibit an earlier wave of development of the adaptive immune system, which supports the generation of a population of T cells with a predisposition to become Treg. In my view this mechanism exists to facilitate the establishment of peripheral tolerance. In addition such a mechanism might aid in suppressing potentially devastating immunity that could be initiated against invading allogeneic cells from the mother and other foreign pathogens and antigens that are capable of crossing the placenta. Such a mechanism has not been described in the mice for the simple reason that the mouse does not export T cells in any appreciable numbers to the peripheral tissues until after birth.[10,11]

Are Treg the Critical Factor Determining Tolerance of Maternal and Fraternal Microchimerism?

CC Anderson, critique. Part of the premise for suggesting that there must be additional mechanisms (beyond central tolerance) controlling immune responses in the human fetus, such as that to maternal allo antigens, is the fact that the human immune system develops much earlier than in the mouse and therefore the authors conclude the tendency toward tolerance cannot just be a result of the "failure to generate adaptive immunity to foreign antigen." The implication is that the human immune system already contains mature cells, for example T cells, prior to exposure to maternal cells. However, I do not see any evidence provided that supports this premise. What evidence is there that maternal cells are not present within the fetus prior to export of this first wave of fetal T cells in humans or any other species?

The data reported by this group in *Science* in 2008, showing increased maternal allo antigen specific responses upon removal of regulatory T cells are certainly of interest, however, there are some problems in interpreting this data as the primary mechanism of unresponsiveness to maternal antigens.[12] While there may be a role for regulatory T cells, the much simpler explanation for tolerance to maternal antigens remains untested in the human setting. This is the concept that maternal cells will induce tolerance via central tolerance in the fetus. The author's argument that this is not the explanation is based on studies using maternal APCs to stimulate fetal T cells. However, tolerance, like immunity, is precisely tuned to the level and type of antigen the T cells are exposed to. Maternal APCs are likely not the same kind of cell as the maternal chimeric cells that are circulating in the fetus. This reviewer's expectation is that the authors would find if they used the truly chimeric maternal cells (maternal cells circulating in the fetus) as stimulators for their T-cell response, that regulatory T cell depletion would not unmask a substantial response of fetal cells to these maternal chimeric cells. That is, the fetus is centrally tolerant to the chimeric cells. They cannot become centrally tolerant to other maternal cells (e.g., APCs) because those cells do not reach the fetal thymus, hence one can unmask a

response to these maternal cells. Maternal APCs express different levels of MHC and different peptides than the circulating chimeric cells. If one wants to understand tolerance to the maternal chimeric cells one cannot assume that studying tolerance to other types of maternal cells is equivalent. Similar reasoning explains why the chimeric cattle twins can, although delayed, reject skin grafts from their fraternal twin. The chimerism induced tolerance specifically to the chimeric cells but not to skin cells from the same donor. The concept of tissue specific antigens would seem to fully explain the data, so it is not clear why the authors consider the chimeric cattle twin experiments need revisiting.

Jeff Mold, response. There is no evidence to say that maternal cells aren't present from the onset of fetal development. Notably the possibility that there are, does not take away from our model. Our model is not inconsistent with the notion that central tolerance could impact fetal T cell development. However we did find that maternal "antigen presenting cells" (here they are T and NK depleted PBMCs) could stimulate $CD8^+$ T cell responses in the fetus, in the absence of Tregs, at a ratio of 1 maternal APC: 250 fetal cells in a five day primary mixed leucocyte reaction (MLR).[12] This is substantially lower than we have seen when using adult T cells and within the range that we detected for the frequency of microchimerism in some of our samples.[12] Colin argues that the types of maternal cells we are using are not identical to the chimeric cells, though we did show in this paper that in the cord blood we were able to observe maternal cells that represented a range of different cells such as those likely to be found in the blood. Nonetheless, the nature of the microchimeric cells in the human fetus is still mostly unknown, and may represent a different type of cell altogether (e.g., a mobile stem cell population) which would obviously complicate the argument and I will concede that it is possible that this could impact our results and the conclusions drawn from them.

With respect to the notion that central tolerance represents a 'simpler explanation' I would have to disagree. For tolerance by deletion to result in long-lasting tolerance to NIMA, as was first described by Claas et al.[13] and clinically noted by Burlingham et al.,[14] one would need to have persistent engraftment of the thymus by maternal cells. Alternatively a mechanism that relied on peripheral tolerance would not necessarily require persistent expression of maternal alloantigens in the periphery. Nonetheless, most individuals do exhibit evidence of low levels of maternal microchimerism in the periphery even into adulthood. While the possibility that maternal cells engraft the thymus at a level that would allow for continued deletion of newly developing T cells throughout childhood has not been ruled out (or tested for that matter), it is my opinion that such a scenario is not 'simpler' than the one that I have offered.

I also believe that the delayed response to a skin graft in fraternal cattle twins would be consistent with the idea that a peripheral tolerance mechanism exists that is simply overpowered in the presence of a large amount of antigens, resident immune cells, and inflammation induced by the surgical procedure. It is known that in mice and other species, that peripheral tolerance can be overcome by increasing antigen dose and applying auxiliary stimulation.[15]

CC Anderson, response. Based on existing data, it can be anticipated that any inductive signals the skin graft generates in the chimeric cattle twin will generate a response that is highly specific to antigens that are present on the skin and not present on the chimeric blood cells of the other twin. The surgically induced signals do not overcome the tolerance to the chimeric cells themselves, the chimerism remains while the skin is rejected.

Jeff Mold, response. Again, in our studies we looked at allogeneic responses to maternal cells and saw no evidence that would suggest that T cells reactive to maternal alloantigens had been purged from the system by central tolerance. This is not by any means evidence against this form of tolerance. A more careful dissection of the adaptive immune responses generated by allogeneic tissue grafts in larger mammals (such as sheep or cattle) would be highly informative for settling such an argument.

How Do We Explain Immunity to Immune Challenge During the Fetal Period and Are There Substantial Differences Between Species?

CC Anderson, critique. Any theory regarding how tolerance is established early in life needs to deal with the fact that the actual experiments, testing whether antigen introduced early in life induces tolerance, are relatively evenly split, with many showing tolerance and just as many showing induction of immunity rather than tolerance. Thus, for this reviewer, the main drawback to the current synthesis of the authors is that their theory does not explain what determines whether antigen introduced early in life will induce tolerance or immunity. Their theory would predict that all introductions of antigen early in life would induce tolerance, contradicting about half the literature in the area, including that of Nicole Le Douarin.[16]

Jeff Mold, response. It is very true that an immense literature exists about tolerance induction in many different species dating back to the 1950s. In recent years, however, work in the mouse model has dominated investigation in this area, and prominent research articles on mouse fetal immune development frequently omit any reference to differences between the mouse and humans.[10,11]

Because the mouse does not develop a peripheral adaptive immune system until after birth, any comparisons regarding intrauterine tolerance are very difficult in mice and humans. Further, the way that the mouse immune system develops appears to differ in many respects that extend beyond simple temporal differences.[9,17] I will elaborate on this with respect to the early work of Nicole Le Douarin below.

We cited the work of Nicole Le Douarin because it is the first evidence that the development of the peripheral immune system occurs in stages, or "waves", rather than as a continuum.[18] This is an important component of our argument for how the human immune system may work during development, as it offers an explanation for how different types of lymphocytes may be generated at different stages of development.[9] This turns out to be true in the mouse as well, with the earliest waves of thymocyte development resulting in the generation of a very specific type of γ/δ T cells (DETC) that only are generated in the thymus

during a specific window of fetal development.[19] In fact, a molecular mechanism underlying the shift from fetal to adult hematopoiesis has now been identified, establishing fetal and adult hematopoiesis as fundamentally different processes with different outcomes.[17]

Notably, in humans, there is no evidence for a bias toward specialized γ/δ T cells developing at the onset of thymopoiesis, or at any stage of fetal thymopoiesis for that matter, arguing that the mature hematopoietic cells produced by these "waves" of thymic output differ between species. We have shown that the earliest waves of thymopoiesis in the human generate α/β T cells (both CD4 and CD8) that behave differently from those found in the adult and seem predisposed to adopting a Treg fate after stimulation.[9] As previously mentioned, we have also recapitulated this scenario by injecting fetal and adult hematopoietic stem/progenitor cells (HSPC) into a fetal thymus from a single donor and showing that the fetal HSPC gives rise to α/β T cells that, while phenotypically similar to their adult counterparts, have a propensity to generate Treg cells upon activation and display a vastly different pattern of gene expression at "resting" state. So this is where the work of Nicole Le Douarin and our work likely differs—at the level of what cells are generated by the timed appearance of different populations of HSC during development.

This difference aside, I do agree with Colin that not all antigens are likely to induce tolerance during fetal development. In fact it is well known that some pathogens which infect the human fetus result in substantial inflammation,[20] and it has been known since the 1960s that introduction of different types of antigens at different stages of fetal development in lambs can lead to tolerance or inflammation[21]—suggesting that additional mechanisms play an important role in dictating outcome. Given our current understanding of immunology (gained through studying the mouse), it would be highly informative to revisit these studies with a greater focus on the mechanisms involved.

CC Anderson, response. The differences between mouse and human are important and should not be ignored, however, it is not clear that the differences impinge on the mechanism(s) of self/non-self-discrimination, their roles lie elsewhere (e.g., immune class control, responses to flora etc.). To develop this point further, differences between mouse and human immune system development are likely to relate to the differences in exposure to flora. In mice, the adaptive immune system is generated at approximately the same time that the mouse begins to acquire its normal flora, during the neonatal period. In contrast, the human adaptive immune system is largely established before any appearance of normal flora. Thus, the human immune system has a much greater degree of competence when it first encounters flora, perhaps necessitating a greater degree of downregulation (e.g., Treg) of particular types of responses.

Jeff Mold, response. The explanation that flora are a critical component of the early Treg response cannot explain the severe autoimmunity in the pancreas and intestines that develops in utero in the absence of a functional FOXP3 protein in severe cases of IPEX.[22] This condition proves that the Treg cells present in the fetus are critical for the self/non-self-tolerance at least toward specific tissues. Additionally, the development of autoimmunity prior to microbial colonization rules out a dominant role for commensals or foreign pathogens in driving this response.

I think it is worth examining the existing data on an animal model (sheep) that is more likely to reflect the human with respect to the temporal development of an adaptive immune system. It has been known for several decades that allogeneic skin grafts transplanted onto fetal lambs up until around 70 d of gestation (the gestational period is 150 d for fetal lambs) are generally accepted, or showed very late rejection, whereas those grafted after 80 d of gestation were universally rejected.[7,21] This does not directly correspond with the development of a peripheral immune system since T cells first appear in the spleen of the fetal lamb at approximately 45 d of gestation and both $CD4^+$ and $CD8^+$ T cells where they increase in numbers thereafter.[23] So then, what can account for a shift from tolerance to rejection of the same type of tissue, transplanted in the same manner, at different stages of development? Presumably each condition leads to chimerism in a similar way and each would result in the same level of thymic presentation of skin graft derived antigens. For me, there are two basic explanations that come to mind. (1) That the timing of rejection reflects a tipping point regarding the maturation of the peripheral immune system reflected by the gross numbers of cells that are present in the fetal periphery at various stages of development. (2) That the timing of rejection reflects a fundamental shift in the type of peripheral immune system that is present at the time of the procedure.

I should point out that more recent work has shown that fetal skin grafts taken before 60 d of development are rejected when transplanted onto allogeneic fetal lambs whereas fetal allografts from older lambs (greater than 65 g.d.) and from adults are accepted.[24] The presumed mechanism involves the introduction of antigen presenting cells to the skin at later timepoints that are required for tolerance. Whether these cells migrate to the thymus to achieve this effect or act on local lymph nodes is disputable and might offer some conclusive evidence regarding the role of central vs. peripheral tolerance in this particular setting. However, I would argue that interactions with fetal T cells and allogeneic APCs present on the skin graft would generate Treg cells that would facilitate tolerance as a primary mechanism.

Another approach has also been employed by the same group to test the role of fetal T cells in generating suppressive tolerance in the developing lamb.[25] Twin fetal lambs were artificially generated by splitting a blastocysts and one twin was subjected to thyroid removal prior to the development of a functional thymus. The twin that lacked a thyroid developed T cells that could react against self-thyrocytes, suggesting that the thymus was capable of releasing T cells with specificity for thyroid autoantigens. Interestingly, this autoreactivity was completely inhibited by T cells derived from the identical twin that was not thyroidectomized. The conclusion was that peripheral antigens were required in the fetus to generate functional tolerance, supporting our model.

CC Anderson, response. Jeff's explanation for the age at which fetal sheep develop the capacity to reject allogeneic skin (explanation 1 above) is fully consistent with the parsimonious explanation that central tolerance is the key determining factor. While

there are a few circulating cells with apparent T cell markers prior to the age at which fetal sheep begin to have the capacity to reject, clearly there are insufficient numbers of T cells or these T cells are not really sufficiently functional α/β T cells at this point. There is no need to invoke a more complex explanation such as explanation 2, "a fundamental shift in the type of peripheral immune system." There will be, a priori, a minimum number of non-tolerant immunocompetent T cells needed to reject a skin graft. Prior to day 70 this minimum has not been met. Fetal recipient exposure to adult donor skin prior to this age leads to graft acceptance because the vast majority of newly generating anti-donor T cells will be made tolerant due to the presence of donor antigen in the recipient thymus; anti-donor T cells cannot accumulate to the minimum threshold number. When grafts are placed into older fetuses, there has not been central tolerance to the donor and sufficient numbers of anti-donor T cells have accumulated to cause rejection. Thus, rejection vs. acceptance of the skin graft by the fetus does directly correspond with the development of a peripheral immune system.

The above scenario raises the question of the mechanism by which donor antigen from a skin graft reaches the fetal recipient's thymus. While it is reasonable to suggest that adult skin might contain APCs that promote tolerance, by going to the thymus or via other mechanisms, there is evidence against this possibility[26,27] In contrast, as we have demonstrated previously, adult allografts of skin, heart, or islets contain passenger lymphocytes (usually T cells), that migrate to the recipient thymus and induce deletional central tolerance.[28,29] Grafts from donors sufficient in DCs but lacking lymphocytes did not induce tolerance and were rejected. I am pleased to see the citation/discussion of McCullagh's important skin graft study, as based on citations it seemed few others have appreciated its significance.[24] Observations related to the establishment of immunity or tolerance during development, such as those of McCullagh, are central to providing a real understanding of self/nonself discrimination. It is mystifying to me how the community of immunologists can ignore such observations (is it a symptom of the inertia toward a valuing of description over synthesis and understanding?).

Our studies of passenger lymphocytes in grafts have pointed to a simple explanation for McCullagh's data. We postulated that the ability of older (day 95 and older) but not young fetal (85 d or younger) donor skin grafts to be accepted corresponded with the amount of passenger T cells in the skin graft.[30] The straightforward explanation for all of this data is the presence vs. absence (or paucity) of central tolerance. Altogether the data point to the explanatory power of Lederberg's model,[31] the first to propose what we now know as central tolerance.

Jeff Mold, response. While I understand how Colin could favor the first explanation that I have offered, I still maintain that the second remains possible. Here, I believe that there is an excellent opportunity to probe both possibilities using modern experimental techniques. A thorough analysis of the different populations of lymphocytes present in the sheep at different stages of development has not been reported, although experimental evidence has indicated that a fundamental shift in the types of lymphocytes present exists between fetal and neonatal sheep.[32]

Additionally, while I believe that the first explanation I offered is well supported by the experimental studies referenced by Colin above, I remain unconvinced that it can explain the studies performed by McCullagh and Chen on how tolerance to the syngeneic thyroid is absolutely dependent upon the presence of a functional thyroid during fetal development.[25] If deletional tolerance was the primary mechanism involved in purging self-reactive T cells, it should be inconsequential whether a peripheral thyroid was present or not. McCullagh and Chen's experimental assertion that a dominant suppressive population of cells develops in the non-thyroidectomized twin fetus is completely in line with explanation number 2, that a tolerogenic population of T cells is involved that generate tolerance to peripheral antigens present in the fetus.

Finally, in the case of the study from McCullagh concerning the acceptance of adult, but not fetal, skin allografts, it remains conceivable that passenger lymphocytes are critical not because they migrate to the thymus but because they migrate to draining lymph nodes where they stimulate the development of fetal Treg cells. In all likelihood a combination of the two is happening, and I would welcome the chance to re-examine this seminal work with modern approaches to address each possibility.

Self/Nonself Discrimination in the Fetus and Species Differences, A Second Look

CC Anderson, critique. The authors highlight an important area, the role of Treg cells, as a contributor to early life tolerance and maintenance of maternal chimerism in the offspring. However, it does not seem to answer why chimerism (i.e., systemic allogeneic cells) is required for allogeneic tolerance to antigens present early in life (or before immune system development). What is the authors' explanation for Nicole Le Douarin's observation that allogeneic or xenogeneic limb buds transplanted into fetal chickens leads to a rejection response against the limb post hatching?[16] Why don't the enhanced fetal Treg block this? In this reviewer's view, the parsimonious explanation for this, and almost all the other data, is that it is primarily the location of allo antigens that determines whether exposure to antigen in fetal life will induce immunity or tolerance. Situations where there is tolerance that occurs as a result of exposure to antigen prior to immune system development are generally those that involve systemic chimerism, and therefore are likely to have induced some central tolerance. While situations where antigen present prior to immune system development induces immunity, are associated with localized antigen (e.g., the limb bud experiments;[16] without the thymus graft).

There are studies from the 1970s to within the last few years examining how a newly generated immune system (the first waves of T cells generated) responds to antigens that preexisted the T cells in the host. For example, skin grafts or other types of transplants given to immune deficient animals (e.g., SCID or Rag-knockout mice) prior to allowing the recipient's immune system to develop through thymus transplantation (in the case of nude mice) or hematopoietic stem cell reconstitution (in the case of SCID/Rag-knockout recipients). These studies indicate that

a newly generated immune system is quite capable of attacking antigens/tissues that were present before the very first T-cell was generated.[33-36] With few exceptions, only if donor antigens reached the thymus was tolerance established.[27-30] These data, not discussed by the authors, are fully consistent with experiments done directly in the fetus, such as those of Nicole Le Douarin and McCullagh.[16,24] Their findings were consistent with the idea that the foreign antigens had to be in the thymus to induce tolerance (e.g., via a donor thymus transplant). Thus, while Treg cells and other aspects of the fetus may have some important effects on tolerance, it seems to me that the primary mechanism of tolerance (central tolerance) to antigens that get into the fetus has been ignored by the authors.

Jeff Mold, response. I am not certain that chimerism is absolutely required in humans early in life to achieve tolerance. However, if we assume that it is, I think that a potential explanation is that Tregs are generated, and that like any other T cells they require the persistent exposure to antigens to be maintained over time.

Simply put, the reason why the chickens reject allo or xenografts is because unlike humans, and like mice, they don't have a Treg response before birth. However there is recent evidence that late stage inoculation (embryonic day 20) of antigens into chicken embryos does lead to a regulatory T cell mediated form of tolerance suggesting that my model does apply to this system in some ways.[37] It is important to underscore, for the purposes of my argument, the real differences between what we have seen in humans and what is known to occur in the mouse and other model organisms. To really illustrate the differences in terms of Tregs, I will reference two studies that have performed rigorous analyses of the temporal development of the Treg lineage in mice and in humans. The first, from Asano et al. in 1996,[38] expands upon the observation that in mice a neonatal thymectomy performed prior to the 3rd day of life results in the development of several autoimmune disorders later in life. They go on to show that this result can be explained by the fact that the very first Tregs appear at approximately the 3rd day after birth in the thymus and expand to 10% of total $CD4^+$ T cells in the following 2 weeks, a frequency at which they are maintained thereafter. In another study by Takahata et al. 2004,[39] $CD4^+CD25^+$ T cells were found to represent 15–20% of the $CD4^+$ T cell fraction at 24 gestational weeks (g.w.) of fetal development, and were found to undergo a linear decline in frequency to 5–10% by 35–40 g.w. around the time of birth. As mentioned before, we and others have noted a sustained frequency of 15–20% CD4+CD25+ Tregs in the period between 12–20 g.w., consistent with the results of Takahata et al. Therefore attempting to extrapolate the model we have developed for humans onto other settings in different species is complicated by these differences.

To really get at these questions we would have to determine a setting in human beings where antigen was present, but limited from reaching the thymus, during the mid-stages fetal development. In addition the central tolerance by clonal deletion argument cannot explain the clinical manifestations of the genetic disease IPEX.[22] It is impossible to know precisely when inflammation began in these cases, yet I suspect it occurs around the time that the "fetal" HSC is overtaken by the "adult" HSC. I think that this occurs sometime in the 3rd trimester and parallels the drop in Treg frequency in the fetal periphery.[39]

There is also specific case where defects in central tolerance occur in human beings. APECED patients with inherited mutations in the AIRE protein, which is responsible for producing a broad range of self-antigens in thymic APCs and medullary epithelial cells, have a distinct deficiency in central tolerance.[40] To my knowledge, there are no examples of fetal demise resulting from AIRE mutations similar to that reported for a subset of patients with FOXP3 mutations. It would be interesting to determine whether such examples exist. In fact the diseases that manifest from these two genetic deficiencies have some overlap but also have distinct features that distinguish them, suggesting that central tolerance and peripheral tolerance are likely to have different effects on immunity.[40]

I do not believe that a newly generated T cell in the adult, even in the situation of a T cell deficient adult, looks anything like a fetal T cell. First, the repertoire of the T cells is going to be dramatically different due to the expression of DNTT in newly made adult T cells.[9,41] Second, in our situation a T cell generated by HSC transplant from an adult donor into an immunodeficient recipient would not behave the same as a fetal T cell.[9] Perhaps if you were to transplant fetal HSC into an immunodeficient human you might be able to directly test this.

Our model, while very preliminary, offers an explanation for why there is a preponderance of Tregs in all human fetuses that declines throughout gestation. We have used the situation of maternal alloantigens as a model to discuss how fetal Treg may impact fetal immunity but I believe that this is not the main role of these cells.[12] The fetus is a rapidly evolving organism with enormous amounts of new cells being generated on a daily basis, in addition to changes in organs that occur at a fairly rapid rate. I believe that a peripheral tolerance mechanism that rapidly adapts to these changes would be a far more efficient system than central tolerance. Especially when you consider the vast numbers of T cells that are generated by the thymus every day (estimated at approximately 10 million new $CD4^+$ cells per day in a 20–30 y old human after involution has begun).[42] Consider, in addition, that during mid-gestation the architecture and cell types of the thymus are also undergoing dramatic changes.[43] The potential that any given T cell may not be educated to all possible antigens that it could potentially encounter in the fetal periphery seems likely to me.

Perhaps the mouse has circumvented this problem by restricting α/β T cell export until after birth.[10] I find it odd that the fetal mouse thymus has been shown to have specific mechanisms in place to prevent T cell export to the periphery a fact that has been assumed to play a role in controlling fetal responses to maternal alloantigens in an allogeneic setting but that clearly doesn't apply to human beings, or many other animals with longer gestations for that matter.[11]

I believe that the role of central tolerance in shaping the peripheral T cell pool is, first and foremost, not incompatible with our model for fetal tolerance development. I believe that central tolerance provides us with a broad range of T cells that

have been purged of many cells that have autoreactive potential. However, even in the mouse it is well known that defects in central tolerance, including AIRE deficiency[44] and BIM deficiency,[45] allow for the escape of numerous autoreactive T cells to the periphery, yet these mice fail to develop overt autoimmunity until later in life. Thus, I would argue that a second system must be in place to ensure that tolerance to all antigens present in the developing fetus develops appropriately. I would argue that there is ample evidence for such a model in organisms in which the adaptive immune system is generated at early stages of fetal development.

CC Anderson, response. To begin, there is no data to argue that the outcome of a pre-immunocompetence allo- or xenotransplant would be any different in humans than in chickens or mice. Irrespective of this important consideration, if indeed the reason chickens reject allo or xenografts given at the fetal stage is that unlike humans they don't have a Treg response before birth, this would indicate that the level of Treg is not central to determining the self/nonself discrimination. The chickens grow up with normal self-tolerance and yet lack the putatively important level of Treg. Thus, the species differences described must not be a key component required for self/nonself discrimination but instead serve some other function.

I agree that a need for central tolerance is unlikely to explain IPEX, just as a lack of Treg is unlikely to explain rejection of pre-immunocompetence transplants, they are simply different problems. Central tolerance, being a key mechanism of self/nonself discrimination explains the latter phenomena, while IPEX points to a need for Treg to either raise the threshold level of signals needed for a response (independent of self/nonself) or in maintenance of general homeostasis. The question Jeff appears to raise is whether Treg might instead be a central component determining self/nonself discrimination similar to the role of central tolerance. To date, there is little evidence to support such a view. Just because a lack of Treg leads to what appears like autoimmune attack does not mean Treg are a discriminatory mechanism in the immune response. Perhaps an analogy would be clarifying. If we wish to discriminate particles that are smaller vs. larger than a particular size using filtration, we would all agree that the mechanism of the sorting (discrimination) is the pore size of the filter. Defects in the pore size will affect the ability to discriminate. Similarly, defects in the attachment of the filter to the structure holding the filter, e.g., a ring around the filter, will prevent the ability to discriminate, but we would not consider the ring as the mechanism of discrimination. What we are discussing is the identification of the mechanism that discriminates whether an antigen expressing tissue will be attacked or tolerated in a growing fetus/neonate.

I do not disagree at all with Jeff's conclusion that peripheral tolerance is a necessary component. Where we seem to disagree is the relative contributions of the various mechanisms to self/nonself discrimination. The observations of Mold and McCune are important. The question remains whether these observations impact on our understanding of self/nonself discrimination in a substantial way, or as an additional fine-tuning process, or in some other immune processes unrelated to the self/nonself discrimination. Only when it becomes clear that a particular observation cannot be explained by central tolerance does it become worth considering additional mechanisms occurring in the periphery.

In our own experiments, where we gave male skin grafts from Rag-knockout donors to Rag-knockout female recipients and immune reconstituted the recipients with wild type fetal (not adult) HSC, these mice appeared fully self-tolerant, and yet they rejected their well healed-in pre-existing male skin graft.[27] Only if the skin grafts had passenger T cells that could generate chimerism and central tolerance was the skin graft tolerated.[28] It will certainly be of interest to examine whether these fetal HSC derived T cells in adult animals express DNTT. However, even if they do express DNTT, the fact that they appear quite competent to make a self/nonself discrimination argues that the primary determining factor in the self/nonself discrimination is the distribution of the antigen; did the antigen get to the central lymphoid organs? Even the presence of hematopoietic chimerism was not sufficient to lead to tolerance in the developing immune system if the chimerism was located only in the periphery and not in the thymus.[29,46]

From our data we concluded that "natural" peripheral tolerance only evolved the capacity to handle a small number of antigens (a small responding repertoire of T cells).[29] Might we have underestimated the importance or capacity of peripheral tolerance mechanisms because of our choice of model system (HSC reconstitution of adult immunodeficient hosts)? It is certainly possible that we have to some degree underestimated peripheral tolerance, as the capacity to establish peripheral tolerance may be reduced in a setting of temporary lymphopenia, a state present in our model. The lymphopenia driven activation that occurs in this setting did not lead to a gross defect in self-tolerance, however, more subtle effects on self-tolerance may have escaped our analysis.

In summary, there are clear and important changes in HSC that occur during development. The role of these changes will be an important area of future investigation. To date there are no compelling data to indicate that features specific to fetal HSC are necessary for a successful self/nonself-discrimination. There is also little reason at this point to believe self/nonself discrimination occurs via substantially different mechanisms in mice and humans. One model that has the potential to test the Mold and McCune hypothesized importance of properties specific to fetal HSC in tolerance is that of Rolink and colleagues. They found that adult bone marrow HSC cause a severe autoimmune like syngeneic graft vs. host disease in sublethally irradiated Rag-knockout recipients.[47] While this may largely be a response to flora, assuming there is a significant autoimmune component, would fetal HSC instead generate a self-tolerant immune system in this setting? Similarly, in our own irradiation free model, where Rag competent fetal HSC do not cause disease in syngeneic Rag-knockout recipients, might purified adult bone marrow HSC instead cause disease? This discussion has rekindled our interest in examining these questions.

Concluding Response

Jeff Mold. As Colin points out, it is difficult to argue that the reason why the chicken or the mouse rejects an allograft given prior to the development of tolerance is due to a lack of Treg cells specific for alloantigens. In the absence of such a mechanism it would appear likely that alternative systems would exist to account for the types of immunological challenges encountered in different species. However, I would argue that the existing data (in mice at least) would suggest that the provision of alloantigen-specific Treg cells would suppress the rejection of the foreign tissue.[48] Why is it incorrect to assume that in the case of humans, where such cells already exist as a consequence of normal development, this mechanism would not be likely to play an important role in the generation of tolerance in utero?

It has been my experience working with human fetal T cells that they are peculiar in their response in a way that makes me believe that they would be incapable of mediating a rejection response in the face of antigenic challenge. Historically this has been regarded as "immaturity" of the fetal immune response in humans. I think that this is an outdated and very oversimplified view, since the term "immaturity" is generally considered synonymous with "non-functional." If we accept this premise, that the fetal adaptive immune system is functional in human beings, we are forced to accept that some peculiarities must exist that explain the differences that are seen between the way that the fetus, neonate, and adult respond to foreign antigens (vaccines, infections, maternal cells).

In the course of my studies on the fetal immune response in humans I confronted these questions with the tools that were available. Unfortunately, these are limited in comparison to those that have been used to study such responses in the mouse. For example, we did not attempt to address the roles of central tolerance in the human fetus, though I am confident of the existence and importance of central tolerance in the human fetus, and I believe that it is likely to play an important role in shaping the peripheral immune repertoire. Our findings pointed to the existence of a specific type of T cell response seen in the fetus that was qualitatively different from what we observed in parallel experiments performed on adult cells, or on neonatal cells for that matter. Whether this response has evolved to supplement the role of central tolerance in controlling the self/nonself-immune response or whether it is playing a specific role in the fetus and is unessential after development remains unknown. For now I can only speculate to the importance. My main hope is that by providing testable models and challenging established views that we can gain a better understanding of the mechanisms that regulated the development of immunological competence and increase our understanding for the advancement of therapies aimed at treating human diseases.

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